The Effectiveness of Methadone Maintenance Therapy (MMT) on Drug Tolerance, Mediated by Receptor Signaling, Signal Transduction and Intracellular Transport

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Article

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Abstract

Introduction: In chronic drug abuse, opioidergic, dopaminergic signaling, and endocytosis are implicated in neural circuit disruption. We evaluate the impact of Methadone Maintenance Therapy (MMT) on opioid addiction.

Method: The expression of dopamine (DRD1- DRD5) and opioid (mu-, delta-, and kappa) receptors, Catechol-O-methyltransferase (COMT), Dynamin 1 like (DNM1L), and RAS-associated (RAB22A) genes in Peripheral Blood Mononuclear Cells from MMT (n= 40) and control (n= 40) were detected by qPCR. Protein-protein interaction (PPI) investigation into addiction-associated genes was performed to elucidate the possible pathways which may have an interference impact on the treatment of addiction.

Results: We found that DRD1, DRD2, MOR, DOR, and KOR expressions increased, and the COMT and DNM1L expressions were decreased in MMT. A complex brain network orchestrated by three stages of interactions I) opioid receptors II) Dopamine receptors III) Intracellular vesicular transport (RAB22A), and synaptic vesicle recycling (DNM1L), involved which led to resistance. We elucidate possible pathways that may have an interfering effect on the opioid use disorder (OUD) treatment, and protein-protein interaction (PPI) studies were performed on addiction-associated genes.

Conclusion: We introduce Mitogen-Activated Protein Kinase (MAPK) signaling as a significant mediator in addiction and represent DRD2 as a potential therapeutic target for OUD. PPI downstream off-target stimulation may involve ERK activation. Thus, the use of novel agonists and antagonists, in combination with ERK inhibitors, may be of particular interest for future research in addiction treatment.

1. Introduction

Addiction is a brain condition defined as a chronic and relapsing psychiatric disorder that causes compulsive drug-seeking behavior without considering negative consequences (e.g., dysphoria, anxiety, and irritability). [1] This has a significant impact on public health and societal costs because addicts are prone to committing crimes and causing accidents. [2] The use of a diverse spectrum of drugs, readily available in various drug markets, has become a burden on the social health and economy. [3] According to the latest report on the annual prevalence of drug use of 2017 from the United Nations Office on Drugs and Crime (UNODC), Iran has an approximately 3.3% rate of worldwide opiate abuse. [4] Methadone is a mu-opioid receptor agonist utilized widely as a first-line cure in OUD, despite its addictive characteristics. Methadone is a promising treatment for opiate addiction functions as selective activation of β-arrestin over G proteins. It has some noticeable properties, including lowering abuse tendencies and decreasing withdrawal symptoms and drug cravings. [5]

Chronic drug abuse disrupts brain pathways, especially those involved in the reward system. These neuronal modifications lead to dependence and tolerance. Three major neurocircuits are disturbed in substance use disorders, including binge/intoxication stage related to basal ganglia, withdrawal highlighted negative affect stage of the extended amygdala, and preoccupation or anticipation stage involved prefrontal cortex. [6] Neurotransmitters and neurotransmitter modulator dysregulation have been observed in the mentioned three stages during substance abuse disorder.

Like heroin, methadone is a full agonist for Mu opioid receptors (MOR). However, it has a longer half-life (approximately 24 h) and appears to decrease the transmission frequency of the human immunodeficiency virus (HIV). Methadone triggers a similar opioid receptor in the brain but with a different pharmacokinetic profile. [7] In OUD, the dopaminergic system is triggered by the opioid system, which includes the MOR, Delta opioid receptor (DOR), and Kappa opioid receptor (KOR). These receptors interact with a large family of endogenous opioid peptides and different drugs. [8] Some studies indicate that alterations in the expression of opioid receptor mRNAs may play a role in neuropathy-like diseases. [9,10] While MOR has a critical role in mediating the rewarding properties of opiates [11], DORs are involved in pain modulation [12] and KOR functions in the neurobiological regulation of addictive states, as well as mood and stress disorders. [13] Methadone stimulates MOR and has some agonist action on KOR and possibly DOR; therefore, it has fewer drug-like effects. [7]

An increasing body of research and evidence-based practice over the past forty years has highlighted the role of the dopaminergic pathway in addiction. [14] Neuroanatomical structures in the Ventral Tegmental Area (VTA), including the mesolimbic dopaminergic system, were significantly impacted by these pathways. [15] These areas contain dopamine, Gamma-Aminobutyric Acid (GABA), glutamate-releasing neurons, and other types of neurons that are known to be involved in addiction. The majority of the neuronal population is Dopaminergic, and approximately 65% are known as the A10 group. Dopamine (DA) projections from these neurons are transmitted to the nucleus accumbens, amygdala, hippocampus, prefrontal cortex, and limbic areas, including the mesolimbic and mesocortical systems. Motivational processes are regulated through the mesolimbic system, while the mesocortical system is related to cognitive and motor functions. VTA-DA neuronal projections are related to reward, motivation, addiction, and some neuropsychiatric disorders. During chronic drug abuse, μ-opioid receptors on VTA-DA neurons are involved in the excitation of dopamine in the nucleus accumbens. [16] This path is induced by methadone through MMT.

The five DA receptor subtypes are termed *DRD1*– *DRD5* and are categorized into D1-like (*DRD1* and *DRD5*), and D2-like (*DRD2*, *DRD3*, and *DRD4*) differ based on sequence homology and pharmacology criteria. [17]

Due to limited accessibility of the central nervous system (CNS) dopamine receptors, previous studies suggest that peripheral blood mononuclear cells (PBMCs) could be an efficient proxy for DA system assessment as well as monitoring CNS pathologies. [18,19] Some functional studies showed that PBMCs biomarkers as mirrors of CNS provide a window in neurodegenerative disorders such as Alzheimer's disease; in a condition that the affected tissue is not directly accessible to evaluation. [20,21] Interestingly, an investigation of the transcriptome level of 79 human tissues using microarray showed PBMCs and CNS have significant similarities in neurotransmitter receptors gene expression. [22,23] In addition to the CNS, opioid peptides including MOR, DOR, and KOR opioid receptors are expressed in some immune-competent cells such as lymphocytes. [24] Therefore, using peripheral blood to investigate the expression pattern of dopamine receptors could mimic its expression profile in the brain. However, *DRD1* and *DRD2* expression levels in peripheral blood cells are controversial. [25]

The expression of *DRD1*, *DRD2*, and other DA receptor subtypes is moderated through gene activation. Specifically, *DNM1L* is a gene involved in the endocytosis process, mitochondrial fission/fusion, and has a significant role in neural transmission. Receptor endocytosis could regulate through clathrin-dependent and clathrin-independent pathways, mediated by Rab GTPase members. *RAB22A* is thought to act at multiple levels in the endocytic path, among the Rab GTPase elements. [26] The *RAB22A* protein is involved in other intracellular processes, such as endocytosis and desensitization of receptors, which are essential in developing addiction tolerance. [27]

In addition, addiction is a complex disorder in which several proteins and molecular pathways are involved. To provide a comprehensive understanding of the accurate mechanism, we utilize protein-protein interaction (PPI), network analysis becomes available for deciphering the complexity of the biological process. Network analysis is powerful tool for predicting and identifying biomarkers and probable therapeutic targets in psychiatric disorders such as addiction through topological properties, including degree and betweenness score among highly-connected proteins (hubs) in the network.

Methadone, as an FDA-approved medication, has weakened activation power of the dopaminergic system compared to morphine, which supports the lower potential for euphoric effects in patients. Unlike morphine, methadone has a higher affinity as an NMDA (N-methyl-D-aspartate) receptor antagonism and could inhibit monoamine reuptake. [28] Moreover, one of the benefits of methadone therapy is attaining antidepressant effects through a mechanism that prevents norepinephrine and serotonin reuptake and modulation of pain transmission. [29] However, methadone demonstrates modest effectiveness during chronic opioid treatment, and long-term relapse prevention has associated the risk of abuse liability. Likewise, it has some side effects such as constipation, nausea, vomiting, and respiratory depression that can be lethal. [30] These reasons directed us towards a comprehensive study of MMT and its direct and indirect targets to investigate ambiguous gaps in the current understanding.

The tolerance mechanism during chronic methadone maintenance remains unclear, and the search for alternative therapeutic strategies for OUD is continuously evolving. This study aimed to assess the molecular characteristics of methadone on mediating the transition from dependence to the tolerance of MMT on a molecular level as the primary objective. We selected a relevant gene list to design an intersection network and conveyed an ontology analysis to identify the most enriched pathway associated with addiction pathogenesis. Our secondary objective was to assess topological quantification and expressions activity by identifying a list of the most pivotal hubs of the signaling pathway components.

2. Material And Methods

We designed a case-control study to assess our study objectives. The group of genes that display the most powerful evidence for positive correlation with addiction pathogenesis was selected based on preceding research and also previous work of our group. [14,27,31,32] Our target Genes were selected based on the directly related receptor signaling of methadone, signal transduction of DA pathway, and genes involved in intracellular signaling of receptor endocytosis. Due to the multi-user manner of the addicted individuals and to reduce the heterogeneity of the subjects and acquire dependable results through synchronized the case group, a long-term MMT approach was performed for the case group.

2.1 Sample Study

We selected our cases from volunteer patients who were addicted to opioids. From the contemporary record of Iran, Drug Control Headquarters reviews the superiority of drug misuse among men is nine times greater than women. Also, in other countries, opioid misuse in men has a higher prevalence than women (nearly 70% of all opioid overdose deaths in 2017). [33,34] Accordingly, primarily based totally on countrywide precedence in this study, all the participants were men. Therefore, we recruited a total of 40 OUD patients, who had a history of more than six- months of heroin addiction, and were aged between 23 and 55 years. We strictly followed our exclusion criteria, as using PBMCs has many challenges. Such as all the participants checked for any Bleeding disorder, low platelet count (<100k), or taking medications that interfere with blood clotting, such as aspirin, non-steroidal anti-inflammatory agents or warfarin, anemia (hemoglobin less than 12.3 mg/dL), evidence of an immune deficiency, such as HIV infection or cancer, taking a medication that affects the immune response within the past month, including oral, intravenous, or injectable steroids. We collected a blood sample and examined the PBMCs of OUD patients. They were detoxifying under methadone hydrochloride oral solution (250 mg, Exir, Iran) treatment once a day for six months. Our controls of 40 healthy individuals aged 23 to 55 years old were selected from the general Iranian population and reported no history of OUD. (Table 1 contains information about demographic characteristics). All the proceedings and clinical research were performed in accordance with the ethical standards and amendments of the Declaration of Helsinki. All participants signed the consent form and were informed about the goal and course of this study. This project was approved by the Ethics Committee (Ethical code:21044) of Legal Medicine Research Center, Legal Medicine Organization, Tehran, Iran.

Table 1

Characteristics of addicted and healthy individuals

	Control (n=40)	Methadone Maintained (n=40)
Age (years, means± SD)	40±4.68	39±5.68
Duration of drug use (years, mean±SD)	-	16±0.24
Duration of methadone maintenance (months, mean± SD)	-	6±1.1
Dose of administered methadone (mg/day)	-	70±10

2.2 PBMC Separation

5mL of peripheral blood samples were collected in the morning before methadone administration from OUD patients for syringe sampling. Blood was discharged into a sterile 15 ml tube containing Ethylenediamine-Tetraacetic Acid (EDTA). Subsequently, the 15 ml tube was placed on a cool flask to transfer to the research institute. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden). The lymphocyte layer was collected and washed three times in Phosphate-Buffered Saline (PBS).

2.3 RNA Extraction and cDNA Synthesis

The total messenger Ribonucleic acid (mRNA) from the lymphocytes was extracted by RNA mini kit (Roach, Germany), and the quantity and quality of the RNA were measured by spectrophotometry. Complimentary Deoxyribonucleic acid (cDNA) was synthesized using cDNA synthesis kit (Fermentas Life Sciences, Germany). Samples of cDNA were stored at -70 °C, and the reference cDNA was also used for further analysis.

2.4 Primer design

Primers for *DRD1* to *DRD5*, *COMT*, *OPRM1*, *OPRD1*, *OPRK1*, *DNM1L*, *RAB22A*, and the housekeeping gene (*β-actin*) were designed using Primer Express software to exclude the amplification of genomic DNA and pseudogenes and to confirm the validity of these primers (http://www.ncbi.nlm.ih.gov/tools/primer-blast/).

2.5 Real-Time PCR Analyzes

75ng sample cDNA was used for PCR qualification in a final volume of 25µL with one unit of Taq DNA polymerase. We used Techne Flexigene PCR cycles (Minneapolis, MN, USA) to amplify target and control genes. The expression of *β-actin*, *DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *COMT OPRM1*, *OPRD1*, *OPRK1*, *DNM1L*, and *RAB22A* transcripts were quantified in samples, using real-time PCR (Rotor gene, Corbett, Germany) and LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche, Germany) with specific primers (Table 2).

Table 2

Primers used in real-time PCR

Gene	Genbank Accession Number	Forward primer (5'→ 3')	Reverse primer (5'→ 3')	Amplicon size (bp)
DRD1	NM_000794.3	TGTTTCCTGTCGCTGCTCATCC	TCTGACACAGCCAAGGAGATGAC	128
DRD2	NM_016574.3	ATCAGCATCGACAGGTACACAGC	TCGTTCTGGTCTGCGTTATTGAG	161
DRD3	NM_000796.3	ACATGCCTACTATGCCCTCTCCTAC	ATTCCAGACTCCACCTGTCACCTC	208
DRD4	NM_000796.3	TTCGTCTACTCCGAGGTCCA	CGCACAGGTTGAAGATGGAG	112
DRD5	NM_000798.4	CCATCCTCATCTCCTTCATTCC	AGTCACAGTTCTCTGCATTCACG	153
COMT	NM_000754.3	CTGGAGGCCATTGACACCTA	GGTTGATCTCGATGGTGATGAG	202
OPRM1	NM_000914.4	CTCCATGATCACGGCCATCA	GTAGATGTTGGTGGCAGTCTTCAT	130
OPRD1	NM_000911.4	AGGCCAAGCTGATCAACATCT	TTGGTCACCGTGTCCCAGTA	151
OPRK1	NM_000912.5	ACCAAAGTCAGGGAAGACGTC	ATCAGGGTGTAGCAGACGATG	152
RAB22A	NM_020673.3	CGATGTAAGAGAAGTCATGGAGAGAG	AGGTTGGCGTCAGTGGATGG	150
DNM1L	NM_001278466.1	GACTTTTGGGCGAACCTTAGAATCTG	CAGGACGAGGACCAGTAGCATTTC	98
β- actin	NM_001101.3	AGACGCAGGATGGCATGGG	GAGACCTTCAACACCCCAGCC	161

2.6 Statistical Analysis

Real-time PCR data were inserted into LinReg software to calculate the mean and individual efficiency and Cycle threshold (Ct) of each sample. The gene expression ratios and *P*-value analysis and unpaired (independent) T-test for *DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *COMT OPRM1*, *OPRD1*, *OPRK1*, *DNM1L*, and *RAB22A* were performed by GraphPad Prism 8 Software Tool. The efficiency of each reaction was determined by LinRegPCR software. Real-time PCR data were analyzed by Rest 2009 and GraphPad Prism 8. The HeatMapper (http://www.heatmapper.ca) was used to identify differentially expressed genes. P-values less than 0.05 were considered significant.

2.7 Protein–Protein Interaction (PPI)

Dopaminergic and Addiction related Markers (Data Set Collection)

The associated genes, including *DRD1*, *DRD2*, *DRD3*, *DRD4*, *DRD5*, *COMT*, *OPRD1*, *OPRM1*, *OPRK1*, *DNM1L*, and *RAB22A*, were considered as the seed proteins to construct the PPI network associated with OUD.

We identified the susceptibility genes associated with Addiction, all genes extracted from other studies on human addiction by PCR, northern blot, in situ hybridization, microarray or RNA-seq, the KEGG database, and based on data mining through previous protein-protein interaction studies, Gene Expression Omnibus (GEO) studies, the EVEX database, and an overview of recent PubMed registered publications, as well. The literature search was performed using keywords: Protein-protein interaction, addiction Gene Network, and Seed proteins. The inclusion criteria for protein-protein interaction part are as follows: 1) original manuscript written in English; 2) gene expression research carried out in human's model associated addiction; 3) research have been accomplished through PCR, northern blot, in situ hybridization, microarray or RNA-seq techniques; 4) research conditioned well matched with our inclusion criteria, however, drug utilization did not ever restrict to at least one sort of drug used simultaneously 5) outcomes satisfying the significance threshold of p-value < 0.05. 6) availability of adequate information to calculate the effect size.

Network Construction

We used data mining to identify the genes associated with addiction. These were subsequently expanded to 331 genes which GeneMANIA (version 3.5.2) determined based on the most related genes to a query gene set using a guilt-by-association approach. Nine PPI networks for addiction were visualized, followed by extraction of the intersection network, using the Cytoscape software (version3.7.2) [35]. Afterward, detection of the clusters (highly interconnected regions) in each intersection network was performed using Molecular Complex Detection (MCODE) (http://baderlab.org/Software/MCODE). The dens proteins in clusters were identified according to the vertex weighting by local neighborhood density and outward traversal from a locally dense seed protein. MCODE clustered the whole network by network cutoff = 2, node score cutoff of 0.2 (K-Core:2), and maximum depth 100. [36]

2.8 Network Analysis

Biological network analysis was performed with CentiScaPe (Version 2.2) based on an undirected Network to screen for hub proteins. [37] Topological characteristics are Degree, Betweenness, Closeness, EigenVector, Bridging, Centroid Value, and Eccentricity. [38] The degree is the number of edges that connect to a node. Betweenness is based on communication flow. Closeness estimates how fast the flow of information would be through a given node to other nodes. EigenVector is a measure of the influence of a node in a network. The bridging is a node centrality index based on information flow and topological locality in networks. The probability of having a node whose function is to generate discrete sets of proteins in a cluster or module in a biological network is interpreted as the centroid value. Moreover, the easiness value of a node to be functionally reached by all other proteins in a biological network introduced the eccentricity. [39] These descriptions are based on network analysis of protein interaction data: an introduction published on EMBL-EBI Training module available online (https://www.ebi.ac.uk/training/online).

By using the Minitab1 17.3.1, to identify the most pivotal nodes based on the centrality parameters, the scatter plot for all nodes in the module were plotted considering The values of (degree and betweenness), (degree and bridging), (eigenvector and betweenness) and (eigenvector and degree).

2.9 Enrichment Analysis

Additionally, we performed an enrichment analysis to a deeper awareness of the biological connection behind the network, using DAVID (Database for Annotation, Visualization, and Integrated Discovery), the functional annotation tool, and retrieved Gene Ontology (GO) terms (for more details, see S1 Table). As a practical annotation tool, DAVID uses context to determine the highly related biological pathway to a gene/protein set.

The GO terms and their p values were created for three lists of molecular function (GOTERM_MF_FAT), biological process (GOTERM_BP_FAT), and cellular component (GOTERM_CC_FAT), respectively. (for more details, see S2 Table). Using Reduce + Visualizes Gene Ontology (REVIGO) (http://revigo.irb.hr/), considering parameters: similarity; “Small (0.5)” and semantic similarity measure; “SimRel” were performed for summarized and visualization of the GO classes. [40]

Ultimately, Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed to determine the intersection network's highest scored biological pathways and clusters.

2.10 The 2nd Extended PPI Network.

All the genes were ranked according to seven topological characteristics, and a total score was calculated for each gene. This gene list was sorted by total score and the seed group's lowest score for the complete score cut-off. Based on this cut-off, a total of 30 genes were selected as the backbone of a new gene list. To gain accurate network analysis, a new gene list is

considered input for the instruction of the new 2nd extended PPI network, using STRING (STRING v10.5). STRING could expand and generate the PPI network by neighbor interactors directly interacting with inputted proteins. Afterwards, we used Gephi (version 0.9.2), for large network analysis of the structure of the PPI networks.

3. Results

3.1 Gene Expression Analysis

Fig 1 shows the comparison of dopamine D receptors (*DRD1* to *DRD5*) and *COMT*, *OPRM1*, *OPRD1*, *OPRK1*, *DNM1L*, and *RAB22A* gene expression ratio peripheral blood lymphocytes among the two groups (MMT and control groups). The expression of dopamine D1 and D2 receptors, which are methadone-maintained subjects, were significantly up-regulated in the MMT group by a mean factor of 5.259 and 3.010, respectively, compared to the control group (*P*-value: 0.0081 and *P*<0.0001). As shown in Fig. 1, dopamine D3 and D5 receptor mRNA expression was significantly reduced (down-regulated) in MMT and reached 0.001946 (*P*<0.0001) and 0.01725 (*P*<0.0001) significance when compared to the control group, respectively. However, the D4 gene expression level was not significant by a mean factor of 0.7315 (*P*=0.2621). *COMT* mRNA expression was down-regulated in MMT (in comparison to the control group) by a mean factor of 0.08464 (*P*<0.0001). Beta-actin expression was similar in all the groups tested (data not shown).

Among opioid receptors, MOR, DOR, and KOR expression levels were increased by a mean factor of 6.520 and 6.717, and 6.277 (*P*-value: 0.001 for MOR and *P*<0.0001 for DOR and KOR), respectively. *RAB22A* gene expression level was not significant by a mean factor of 1.398 (*P*=0.3884), and the *DNM1L* gene expression level was decreased to 0.04 (*P*≤ 0.0001).

Fig.2 shows the heat map diagram of expression of 11 genes (shown in x-axis) corresponding to MMT (n=40) and controls (n=40) samples. The open green color represents maximum DeltaCts values, while red is minimum. As shown, *DRD3* and *DRD5* are related to lower gene expression values (higher DeltaCts), while these genes are in control samples with higher-medium expression values (lower DeltaCts). Therefore, the visible detection of the heat map suggests a potential difference between the two groups.

Table 3 represents the results of re-analyzing data in the study groups of OUD patients, with methadone-maintained subjects whose duration of drug usage was in a similar range. Table 3 shows that the MOR, DOR, KOR, and dopamine D1 and D2 receptor mRNA expression were significantly up-regulated in OUD patients, with methadone-maintained, subjects. Dopamine D3 and D5 receptors and *DNM1L* gene and *COMT* enzyme expression were significantly down-regulated in methadone-maintained group subjects. Still, no significant change was seen in the D4 receptor and *RAB22A* mRNA expression level in the methadone-maintained group compared to control subjects.

Table 3

Results of analyzing data related to methadone maintenance treated (MMT)

Gene	Md±SEM	95% CI		Fc	*P*-Value	Expression Result
		Lower	Upper			
DRD1	4.259±1.566	1.140	7.377	5.259	0.0081	UP
DRD2	2.010±0.4072	1.199	2.820	3.010	<0.0001	UP
DRD3	-0.9981±0.1822	-1.361	-0.6353	0.001946	<0.0001	DOWN
DRD4	-0.2685±0.2377	-0.7416	0.2047	0.7315	0.2621	
DRD5	-0.9828±0.1908	-1.363	-0.6029	0.01725	<0.0001	DOWN
COMT	-0.9154±0.1580	-1.230	-0.6008	0.08464	<0.0001	DOWN
OPRM1	5.520±1.619	2.297	8.743	6.520	0.001	UP
OPRD1	5.717±1.048	3.630	7.804	6.717	<0.0001	UP
OPRK1	5.277±1.279	2.732	7.823	6.277	<0.0001	UP
RAB22A	0.3978±0.4586	-0.5153	1.311	1.398	0.3884	
DNM1L	-0.9556±0.2331	-1.420	-0.4916	0.04444	≤ 0.0001	DOWN

Md: Means Differences between two groups (controls & MMT); CI: Confidence Interval; Fc: Fold change. Significant value was less than 0.05 ($P<0.05$)

3.2 Network Analysis

We performed data mining to identify critical factors in biological processes using our gene selection and our raw gene sets (312), (S1 Table), which is expanded during gene ontology enrichment analysis by GeneMANIA (version 3.5.2) (331 genes, S3 Table).

After removing Homo sapien interactions with GeneMANIA in Cytoscape, 331 nodes and 13038 edges were removed in the whole addiction network. (for more details about the Gene list, see S3 Table). Cytoscape platform identified the most related region with the higher connection of the intersection network applied by the MCODE plugin. Nine clusters were detected, as shown in Fig 3. The number of nodes in clusters 1, 2, 3, 4, 5, 6, 7, 8 and 9 was 35, 34, 42, 39, 37, 45, 21, 27 and 5 respectively (score: cluster 1: 21.353, cluster 2: 20.909, cluster 3: 14.634, cluster 4: 13.421, cluster 5: 9.111 ,cluster 6: 8.773 , cluster 7: 5.900 , cluster 8: 5.308 and cluster 9: 3.000), Table 5 details these results. We utilized the Molecular Complex Detection method (MCODE for short) to recognize networks clustering using the gene set extracted from the data mining. MCODE: Table 4 shows the numbers of nodes and edges derived from data mining for the association of Addiction genes. MCODE plugins identified the most important modules in the whole network by each cluster's score. Based on table 4, cluster 1 with a score of 21.353, which consists of dopaminergic and opioidergic seeds, is the most significant module. Within each cluster, the DAVID annotation system was used to determine the most significant KEGG pathways. Table 6 summarizes the results and shows that the highest score cluster (Cluster 1) consists of nodes associated with dopaminergic synapses. Most clusters contain nodes that associate the PI3K-Akt signaling pathway and MAPK pathway (hsa04010).

Table 4

PPI data derived from data mining for Addiction gens associated; MCODE clusters.

Clusters	Node	Edges	Score
1	35	550	21.353
2	34	544	20.909
3	42	738	14.634
4	39	343	13.421
5	37	207	9.111
6	45	227	8.773
7	21	64	5.900
8	27	73	5.308
9	5	7	3.000

Table 5

MCODE clusters nodes.

MCODE Clusters	Node IDs
1	*GNB4,ATP5B,GNB3,GNGT2,PNOC,CCT7,GNAQ,GNG12,DRD5, OPRL1,GNG13,GNG2,GNG8,GNG4,GNGT1,CCT2,DRD1,ATP5G3,OPRD1, LDHB,GNG5,MDH2,DRD4,GNG10,GNG7,CCT5,GNAI1,GNG11,DRD3, OPRK1,ADRA2A,OPRM1,TPI1,DRD2,GNB5*
2	*FZD4,STIP1,ATP5C1,VDAC2,PDHB,PGK1,GOT1,ENO1,HSP90AA1,SDHA,UQCRC1,DLD, COX5B,PARK7,PFKM,HSPA8,CCT4,HSPA9,ATP5A1,VDAC1,SUCLG1,PDHA1,GAPDH, CS,STXBP1,ACO2,TUBA4A,HSP90AB1,GLO1,ATP5G1,NDUFS2,NME1,LDHA,HSPD1*
3	*DPYSL2,NEFL,CSTB,ENO3,DLG4,NME2,CAMK2A,GNB1,GFAP,PPP3CA,HSPA5,IDH3G, GRIA2,ENO2,YWHAB,ACTG1,TUBB2A,EGR4,HTR2A,STX1A,MAP2K1,ERP29,PLCB1, ERAL1,ATP6V1A,EEF2,EGFR,PRKCG,TUBA1A,TPM3,TMOD2,RPS6KB2,ARRB2,MAPK1, GNB2,TUBB3,SYN2,SNCA,CDK5,P4HB,CALM1,SNCB*
4	*FABP7,DRP2,IDH3A,ACTR2,TCP1,GPI,ATP6V1B2,NDUFA10,PPP2R1A,SOD1,PRDX5, ALDOA,ACTB,OGDH,YWHAE,MDH1,NSF,YWHAQ,EEF1A1,TKT,CCT3,PHB,PPIA,GLUD1, PRDX6,YWHAZ,GNAO1,DLAT,HNRNPA1,GRIA3,SNAP25,PGAM1,CAPNS1,NDUFV2, PRDX2,GRIN2A,PKM,GNG3,CAMK2B*
5	*GDI1,CRMP1,AKT1,GRIA4,WDR1,CLTC,PGM1,SLC17A6,HK1,GRIN1,GRK2,HSPA2, SLC9A3R1,TFRC,PEBP1,NDUFA5,CKMT1B,KIF5A,SNPH,PDCD5,ATF2,ALDOC,COMT, GRIN2B,PRKCA,PRKAR1A,PFKP,APP,CAPZB,EEF1G,UBE2N,DNM1L,DNM3,KIF5B, NAPA,SH3GL2,ATP1A1*
6	*ATP1A3,PRKCB,GSTO2,RAB5A,PPP2R2A,PRR11,TAS1R1,WLS,QKI,NRG1,GLUL,CALM2, DNM2,ITPR1,VEGFA,PPP2R5B,GSTO1,SOD2,IL15,ARRB1,ALDH6A1,ANXA5,LRRFIP1, HIF1A,JUN,GSTP1,ATXN1,TPM1,UCHL1,BDNF,RABGEF1,GRIA1,RAB1A,GDA,PPP3R1, NGF,TCEB3,KMT2A,PDIA3,GSTM1,GRIP1,ATP6V1H,ABAT,TRPC1,DDAH1*
7	*GRK4,PPP2R3A,NR4A1,EGF,FLNA,NCS1,SLC6A3,TF,TNF,CALB2,PLD2,CKB,FOS,FSCN1, PCBP3,EEA1,CRYM,GPX1,CTSD,ARF1,ANXA3*
8	*ELK1,IL6,ABCB1,MAOB,CNDP2,PDGFRB,ADIPOQ,LPP,IGF1,PLCXD3,NFKB1,RB1,ITPR2, ALDH5A1,IL13,IL2,PLG,PTPRF,PHGDH,EPHB2,DTX3L,TACR1,CALB1,ICAM1,UBE2L6, CYP19A1,PRL*
9	*NTF3,IL4,SLC6A4,IL1B,NR4A3*

Table 6

KEGG pathways of intersection network and each MCODE cluster by DAVID.

	Number of Nodes	KEGG Pathway	Genes	*p* value
Cluster 1	21	Dopaminergic synapse (hsa04728)	*GNAI1,GNAQ,GNB3,GNB4,GNB5,GNG10,GNG11,GNG12, GNG13,GNG2,GNG4,GNG5,GNG7,GNG8,GNGT1,GNGT2, DRD1,DRD2,DRD3,DRD4,DRD5*	9.2E-28
Cluster 2	8	Alzheime's disease	*ATP5A1, ATP5C1, ATP5G1, NDUFS2, COX5B, GAPDH,SDHA, UQCRC1*	1.4E-5
Cluster 3	7	PI3K-Akt signaling pathway	*GNB1,GNB2,EGFR, MAPK1,MAP2K1,RPS6KB2, YWHAB*	8.2E-3
Cluster 3	6	MAPK signaling pathway	*ARRB2, EGFR, MAPK1,MAP2K1,RPKCG,PPP3CA,*	9.5E-3
Cluster 4	3	Synaptic vesicle cycle	*ATP6V1B2,NSF,SNAP25*	3.5E-2
Cluster 4	5	PI3K-Akt signaling pathway	*GNG3,PPP2R1A,YWHAE,YWHAQ,YWHAZ*	7.9E-2
Cluster 5	10	Endocytosis	*GRK2,SH3GL2,CAPZB,CLTC,DNM1L,DNM3,HSPA2,KIF5A,KIF5B,TFRC,*	5.3E-7
Cluster 6	6	MAPK signaling pathway	*JUN,ARRB1,BDNF,NGF,PRKCB,PPP3R1*	9.5E-3
Cluster 7	5	MAPK signaling pathway	*FOS,EGF,FLNA,NR4A1,TNF*	1.9E-3
Cluster 8	6	PI3K-Akt signaling pathway	*IGF1,IL2,IL6,NFKB1,PDGFRB,PRL*	8.6E-3
Cluster 9	2	Inflammatory bowel disease (IBD)	*IL1B,IL4*	3.9E-2

3.3 Enrichment Analysis

Three GO networks were generated using the enrichments analysis results to identify the most pertinent GO terms (Fig 4). The GO terms data with parameters: more miniature EASE Score (a modified Fisher Exact *p*-value) extracted from DAVID annotation system based on previous enriched gene list which is highly connected in the intersection. According to the p values, the most enriched GO terms related to various biological processes were: response to nitrogen compounds (GO:1901698), behavior (GO:0007610), learning or memory (GO:0007611), enzyme binding (GO:0019899), myelin sheath (GO:0043209), vesicle (GO:0031982) and extracellular exosome (GO:0070062), which are represented in dark red nodes in Fig 4.

3.4 Identification of Hubs

Scoring based on expression-activated subnetwork by the Cytoscape plugin, jActiveModules revealed the total number of nodes in the most expressed active subnetwork (Score: 10.649) consisted of 331 nodes and 13038 edges that were examined for centrality parameters (for more detail, refer to Table S4). Hub nodes with the highest degree and betweenness value, Bridging and degree value, Betweenness and EigenVector value, EigenVector and degree value were demonstrated in a scatter plot of two topological parameters. Fig 5 and Table 7 display these results, while Table S5 provides more detail.

Table 7

Hub nodes.

Node Name	Description	KEGG pathway	Betweenness	Degree	Bridging	EigenVector
GNB1	modulator or transducer in various transmembrane signaling systems.	**PI3K-Akt signaling pathway**	1369.269	176	3.303171	0.098229
APP	triggering caspase activation and degeneration of both neuronal cell bodies	Alzheimer's disease	1040.938	154	3.439378	0.094883
GNAI1	transducers downstream of G protein-coupled receptors (GPCRs)	**Dopaminergic synapse**	1088.623	137	3.873269	0.078279
YWHAB	blockage of neuronal apoptosis	**PI3K-Akt signaling pathway**	1150.104	200	2.554592	0.119928
EGFR	Receptor tyrosine kinase binding ligands of the EGF family	**MAPK signaling pathway/ PI3K-Akt signaling pathway**	1025.011	180	2.584324	0.104659
JUN	increased steroidogenic gene expression upon cAMP signaling pathway stimulation.	**MAPK signaling pathway**	969.597	138	3.407566	0.073867
PPP3CA	calmodulin activation of calcineurin. Dephosphorylates DNM1L, HSPB1 and SSH1	**MAPK signaling pathway**	1029.959	180	2.540511	0.102506
SNCA	regulation of dopamine release and transport. Reduces neuronal responsiveness to various apoptotic stimuli,	Alzheimer's disease	917.7574	167	2.570957	0.098327
DLG4	synaptic plasticity associated with NMDA receptor signaling.	Glutamatergic synapse	890.4552	165	2.667097	0.090502
YWHAZ	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signaling pathways.	**PI3K-Akt signaling pathway**	913.6191	201	2.013213	0.112496

Table S5 ranking displays the hub nodes with the highest degree and betweenness within the network. It was remarkable that three and four out of ten hubs related to MAPK signaling pathway and PI3K-Akt signaling pathway, respectively.

3.5 The second extended PPI network.

Most relevant nodes with the highest score parameters in the intersection network generated a new gene list as input for constructing the second extended PPI network associated with addiction applied by STRING. The second extended PPI network, with co-expression interactions, is displayed in Fig 6. This network demonstrated five nodes for the KEGG pathways of Rap1 signaling and Dopaminergic synapse. This network, without co-expression interactions, revealed ten nodes for the KEGG pathways of Dopaminergic synapse (hsa04728) (*GNB1*, *GNB2*, *GNAQ*, *DRD5*, *PLCB1*, *PPP3CA*, *ATF2*, *GRIN2B*, *GRIN2A*, *GNAI1*), six nodes of Rap1 signaling pathway (hsa04015) (*GNAQ*, *EGFR*, *PLCB1*, *GNAI1*, *GRIN2B*, *GRIN2A*), and other pathways (Table 8).

Table 8

The proteins in the second extended PPI network involved in the KEGG pathways of addition

	KEGG Pathways		
Pathways	*Description*	count in gene set	*false discovery rate*
hsa04728	Dopaminergic synapse	10 of 128	5.57E-12
hsa04724	Glutamatergic synapse	9 of 112	4.87E-11
hsa05030	Cocaine addiction	6 of 49	2.38E-08
hsa05031	Amphetamine addiction	5 of 65	2.29E-06
hsa05034	Alcoholism	6 of 142	3.10E-06
hsa04015	Rap1 signaling pathway	6 of 203	1.65E-05

4. Discussion

4.1 Receptor Signaling

The first gene group that showed differences between control and MMT groups belonged to Receptor Signaling via the opioid receptors (the binding site for morphine and its components). Preclinical experiments increase KOR and MOR mRNA expression in NAs after morphine exposure. [41]Also, preclinical and clinical experiments report that chronic cocaine and amphetamine treatments increased MOR expression. Upregulation of MOR with morphine treatment was detected in Human and Monkey immune cells. [42] Moreover, upregulation of MOR has been reported in methadone maintenance patients. [43] KOR upregulation has been reported in the immune and brain region of rats under chronic morphine treatment. [44,45] Our results show upregulation of MOR and KOR in MMT, which agrees with these previous reports. This is not surprising since the elevation of MOR and KOR expression in addiction has been reported in previous preclinical and clinical studies. [46,47] However, there are contradicting results of Kappa, Mu, and delta receptor downregulation in PBMC during methadone maintenance on heroin-addicted subjects. [48] Coordinately, MMT increased the expression level of DOR in this study. To our knowledge, there is little information for Delta OR mRNA alteration in morphine components induction. Although, the morphine-induced upregulation of DOR cell surface via selective agonist for MOR has been confirmed. [49], another study showed no effect of prolonged morphine on DOR protein level and suggested that the increased DOR density on the cell membrane is due to intracellular receptor reserve. [50] In an animal experiment, no significant difference was detected in DOR mRNA levels between morphine treatment and controls; therefore, no receptor neosynthesis mechanism was observed during morphine induction. [51] We assumed that methadone as a MOR agonist could upregulate the MOR and KOR. On the other hand, DOR upregulation could be mediated via MOR in chronic drug exposure based on previous reports. since heterodimerization of mu and delta-opioid receptors is involved in morphine tolerance [52] and previously in vivo functional study has been reported the co-expression of these receptors in brain areas located in circuits related to drug reward [53] likewise, reduced stress induction in the presence of Delta opioid agonists have been observed during withdrawal in rodents. [12]MOR and DOR agonists inhibited GABA interneurons in the hippocampus resulting in increased pyramidal cell activity, facilitating learning and memory processing through drug use. [31] These findings parallel our results that the opioidergic pathway is activated after long-term methadone abuse, mimicking the OUD process. We also detected upregulation of KOR expression in our drug-abuse cohort. Conversely, another report demonstrated that the expression level of the KOR mRNA in PBLs was significantly decreased in MMT and drug abuse groups [43], which contrasts with our results. We suggest that the elevation of KOR expression may be related to methadone acting as an unspecific agonist for the KOR receptor. Therefore, KOR antagonists may function as pharmacotherapeutic agents by reducing drug craving and consumption. We assumed that one of the compensative mechanisms to overcome receptor desensitization is to extend the ligand's contact area and enhance the sensitivity to the lowest amount of ligand. In turn this mechanism develop tolerance and leads to displaces of

the stimulation threshold, which demands more and more ligands. However, we could not differentiate that this Simultaneously increasing of opioid receptors related to methadone effect as an agonist or retained from chronic drug abuse must be assessed in future study. Moreover, further study is needed to clarify this finding at the protein level.

4.2 Signal Transduction

The excitation of dopamine in the nucleus accumbens could be through μ-opioid receptors on VTA-DA neurons. [54] The activation of the VTA-DA neurons of the limbic system provides a better understanding of the brain circuitry involved in reward, motivation, addiction, and neuropsychiatric illness.

The *DRD1* gene expression level increased significantly under methadone treatment in the present study. According to several reports, increasing D1 dopamine receptor expression and activity in key brain regions is essential for reward and may contribute to drug dependency. [55-57] Glutamate release in the pyramidal cells of the basolateral amygdala (BLA) has mediated by the presynaptic D1 receptor overexpression, suggesting that synaptic transmission may switch from inhibition to excitation in chronic morphine treatment. [58] therefore, our results are in agreement with these findings.

Several addiction studies have reported *DRD2* reduction. [59-61] However, there is a contradiction since upregulation of the *DRD2* gene in opioid abusers has also been observed. [62] However, they assessed the expression level of *DRD2* protein in VTA of postmortem brain of OUD patients and assumed that *DRD2* elevation during chronic opiate abuse could increase the reward. [62] Our results disagree with their finding, and one reason could be that they investigated the VTA region. We assume that an increased level of *DRD2* could contribute to the acute phase of drug abuse. During the chronic phase and adaptation development, expression alteration will occur. Several efforts have been made to use DA agonists as treatment approaches in addiction (in vivo & in vitro). The results suggest reduced self-administration of the drug by overexpression of DA receptors. [59,63-65] Moreover, peripheral D2 receptor antagonists would escalate impulsive behavior. [66] Compulsive behavior has been observed in mice with D2 receptor knockdown in the striatum, representing an addiction-like phenotype. [67] However, overexpression of the D2 receptor appears to attenuate alcohol consumption and cocaine self- administration. [68,69] Furthermore, similar to dopamine transmission, drug administration demonstrates reduced D2 receptor expression level in the animal model (high or low impulsivity). [70] In the present study, we demonstrate the significant increase in *DRD2* gene expression level under methadone treatment. In opiate abuse, MOR regulates DA release in the dopaminergic synapses. Afterward, DA binds to the Dopamine receptors and *DRD2* as well. *DRD2*, an inhibitory GPCR, activates the punishment pathway by inhibiting the indirect ventral striatal signaling through decreased intracellular cAMP. [71] Functional study on OUD patients showed that reduced *DRD2* expression correlated with relapse and drug-seeking behavior. [71,72] Despite *DRD1*, *DRD2* has a high affinity for DA binding. Therefore, a low level of DA could have occupied the *DRD2*, in addiction during firing, DA release following drug abuse, the excitatory pathway through *DRD1* induces. On the other hand, reduction in *DRD2* expression mediates dysregulation of motivation and in accompanied by craving behavior. As previously reported, postsynaptic overexpression of *DRD2* in NAc in mice showed increased motivation. [73] Due to incapability for experience rewarding in low availability of *DRD2* in striatal, the high threshold is demanded. [72] This preliminary evidence represents tolerance development. Ultimately increased *DRD2* expression under methadone maintenance may consider as the positive effect of methadone as the first cure line of addiction (FDA approved agonist) in modulating the DA receptors expression.

Bromocriptine, a *DRD2* agonist, was investigated for drug abuse treatment. Bromocriptine is an agonist (*DRD2*) and a partial antagonist (*DRD3*). [63] functional study in morphine-dependent mice showed that bromocriptine could function as morphine analgesia and inhibit tolerance development; however it promotes opiate withdrawal signs. [74] Although full agonists such as bromocriptine reduce drug self-administration and relieve drug withdrawal symptoms, utility is limited by high abuse liability. [75] Therefore, continued investigation regarding any efficient *DRD2* agonist with a reduced side effect in the long term is needed for future study.

Dopamine D3 receptors are located mainly in the nucleus accumbens, VTA, and amygdala, representing dependence paths in the brain. Moreover, pharmacological, human post mortem, and genetic studies have been supported the role of *DRD3* in drug dependency. [76] In the present study, the mRNA expression level of *DRD3* was downregulated in the MMT group. One probable strategy could be utilizing *DRD3* ligands to reduce relapse in the abstinence stage since the limited direct involvement *DRD3* in drug reinforcement. [76] however, Preclinical data indicate an up-regulation of *DRD3* expression following exposure to DA-elevating drugs, including cocaine, nicotine, alcohol, methamphetamine (MA) polydrug users, and methadone maintained subjects. [77] Preclinical studies demonstrate the beneficial effect of *DRD3* antagonists in decreasing motivation to self-administer drugs and diminishing craving. [77] in agreement with previous functional evidence, we assumed that downregulation of *DRD3* in the MMT group highlights the effects of methadone in decreasing relapse.

Dopamine D4 receptor is expressed in the striatum, cerebral cortex (CTX), and hippocampus where neuronal functions are not well defined. [78] A significant reduction has been reported in dopamine D4 receptor mRNA expression in abstinent, as well as in heroin and alcohol. However, in OUD undergoing MMT, *DRD4* reduction was insignificant, the same as our study group. Thus, it seems that the reduction of dopamine signaling through the dopamine D4 receptor is a risk factor for drug dependency. [78] Functional study by *DRD4* blockade demonstrated attenuated craving and nicotine-seeking behavior. Therefore, *DRD4* antagonists deduce to be effective therapeutic agents to reduce relapse in tobacco addiction. [79,80] Considering these findings, we suggest a potential role for *DRD4* as a novel strategy for treating drug dependence. Hypermethylation of *DRD4* has been observed in heroin abused, which resulted in the *DRD4* receptor expression level alteration in the addicted subjects. These results are considered evidence for *DRD4* role in drug addiction pathophysiology. [81] Dopaminergic deficiency involves in the pathophysiology of depression. A significant reduction of *DRD4* mRNA in PBMC belonged to the patients with major depression was observed. [82] Recently, a meta-analysis study in Iran and around the world was performed and showed the positive effect of methadone on depression in OUD. [83] Our data show no significant difference in the peripheral blood lymphocytes of MMT and control group. According to our results and a similar previous study. [78] We assume that methadone could positively impact the *DRD4* receptor by restoring the expression level and attenuating the significant differences with the control group. So, as a result, one of the benefits of methadone treatment could be modulating depression in OUD, and *DRD4* antagonists could be considered the target of future studies. However, this suspicion is also raised that this result may be because this receptor's naturally low expression level in PBMCs impeded detection or simply that methadone did not impact the *DRD4*.

The data demonstrated a reduction of *DRD5* mRNA expression level in MMT compared to the control group, which supports the pivotal rule of *DRD5* in behavioral patterns connected to OUD. Overall, this data supports increasing responsiveness to drugs and hypersensitivity, congruent to dependence and tolerance strategies. There are some discrepancies between the literature regarding *DRD5* expression in OUD. Significant downregulation of the *DRD5* has been reported in computer game addicts. Also, increased *DRD5* and signaling through this receptor decrease responsiveness to cocaine. [84] However, increased *DRD5* gene expression and activation pathways (G-proteins) have already been described as modulators of OUD. [85,86] In another report, the *DRD5* expression level in OUD subjects and the MMT cohort was not statistically significant. [78] This discrepancy seems to be attributed to brain location-dependent *DRD5* expression, as *DRD5* expressed significantly in the prefrontal cortex and NAc compared to the hippocampus in OUD patients. [87]

The *COMT* enzyme regulates dopamine degradation in the brain. It has a pivotal rule in DA turnover, and we assumed that any changes in the expression level of this enzyme would impact the rate of DA degradation. Furthermore, the reduction of *COMT* could elevate DA levels in extracellular areas. Therefore, *COMT* reduction is a rational process after methadone treatment, which could be a compensating mechanism in DA depletion conditions to avoid withdrawal symptoms.

4.3 Resistance related to Intracellular Transport

DNM1L encodes a large GTPase dynamin 1 like protein, which has an essential role in regulating postsynaptic clathrin-mediated endocytosis and mitochondrial fission/fusion. significant up-regulation of *DNM1L* gene expression in the CTX of animal models for alcohol dependence and microglia exposed to cocaine have been reported previously. [88,89] However, our research group reported that *DNM1L* gene expression was not significantly affected by treatment with methadone and DAMGO in addiction. [27] Downregulation of miR-331-5p, which targets *DNM1L*, has been reported in ketamine abusers. [90] Also, upregulation of *DNM1L* led to altering mitophagy in T and B cells and Auto-antibody production in Systemic Lupus Erythematosus. [91] Upregulation of *DNM1L* was associated with rheumatoid arthritis (RA). *DNM1L* upregulation by induced ROS productio causes oxidative stress. *DNM1L* inhibitor was used for RA. [92] *DNM1L* overexpression associated with proliferation, invasion and, apoptosis in gastric adenocarcinoma. [93]

In the present study, we demonstrate that the mRNA expressions level of the *DNM1L* gene is decreased in MMT patients. *DNM1L* plays a role in synaptic vesicle recycling and is pivotal in receptor internalization pathways. Reduction of *DNM1L* could disrupt synaptic transmission through mitochondrial fission/fusion imbalanced and caused neural activity impairment. [94] We suggest that this reduction of *DNM1L* could disrupt the DA or opioid receptor desensitization due to long-term drug abuse and impairment in synaptic transmission. As previously reported that mitochondria depletion could lead to axonal mitochondria depletion, axonal degeneration, and neuronal death. [94]

Rab proteins are Ras-related GTPases that have a pivotal role in regulating different endocytic steps and maintaining several cellular homeostatic pathways. [26] A study on rapid induction of tolerance by opiate suggested that morphine promotes MOR endocytosis which can cause desensitization and ultimately induces morphine tolerance and dependence. *RAB22A* is one of the downstream components in this endocytosis network. [95] Recently, we reported *DNM1L* gene expression was not significantly affected, but *RAB22A* gene expression decreased with methadone treatment. Thus, the *DNM1L* gene could be involved in cellular pathways of morphine-induced tolerance, and it could explain the difference between morphine and other similar Mu opioid receptor agonists. [27] *RAB22A* gene overexpression has been reported in different human cancer types such as Breast, Pancreatic, Lung, Osteosarcoma, which have a significant part in autophagy, invasive behavior, migration, and migration tumor development through vesicular trafficking and exosome formation. [96-99] recently, many research types have focused on *RAB22A* inhibition using miRNAs to prevent tumor cells from progression and metastasis. However, to our knowledge for the first time, the present study assessed the *RAB22A* expression in OUD patients. The present study demonstrates that the *RAB22A1* gene expression in MMT patients was not significant. During the treatment period, methadone may moderate *RAB22A* in the MMT group, which could normalize the activity of *RAB22A*. [100] Alternatively, *RAB22A* mRNA level could compensate for *DNM1L* expression reduction to overcome tolerance development.

4.4 Protein–Protein Interaction (PPI)

We assess three protein groups involved in different steps, including receptor signaling, signal transduction, and resistance. Generally, in system biology, proteins functions in a complex, not separately. In the same cellular process, all the participating gene products interact. The PPIs study could elucidate the function of each protein within the cell. One of the advantages of PPIs study is that the undefined function of the protein could be predictable according to the other related proteins' function. [101] The accurate exploration of PPIs facilitates the identification of functional pathways to accelerate the molecular mechanisms underlying cellular processes. Therefore, with the help of PPI, we assess the relation between these three groups and highlight the other link protein and pathways that are potentially involved in OUD pathophysiology.

To illustrate any connection between addiction-associated genes, this investigation constructed the PPI networks and provided clustering and enrichment analysis. MAPK signaling pathway demonstrates the most significant enriched pathway within the highest scored cluster during ontology analysis of the intersection MCODE clusters. The enriched GO terms corresponding to cellular components show myelin sheath as an impacted area (GO:0043209)(Fig 4). The speed and efficiency of neuronal communication depend on myelination's degree. [102] However, there are areas of demyelination in

different brain regions in alcohol and tobacco users[103,104]. Decreased myelination could contribute to impulsivity behavior in OUD remains unclear and require further research and testing. Behavior (GO:0007610) and learning or memory (GO:0007611) are highlighted for biological processes. MAPK-ERK signaling pathway plays a critical role in generating long-term stable alterations underlying learning and memory in addiction. [105] As shown in (Fig 5), most hub nodes demonstrate targets that have a pivotal role in the MAPK-ERK signaling pathway.

Methadone is used as a usual medication for pain relief and drug addiction. However, long-term MMT has some disadvantages as well. A better understanding of dependence and tolerance during MMT is revealed by linkages between the most critical hubs and their involved pathways extracted from the theoretical network analysis and the experimental section results and clarify the advantages and disadvantages of methadone as a therapeutic strategy. In long-term MMT, methadone (a full agonist of opioid receptors) binds to the opioid receptors, particularly the μ-opioid receptor (MOR). And stimulates intracellular downstream signaling, including adenylate cyclase-cAMP/protein kinase A. In the acute phase of opioid abuse, MOR decreases gamma-aminobutyric (GABA) release in VTA and triggers DA release in NAc. [106] Instead, in chronic opioid use, increasing GABA release in the synaptic cleft of VTA occurs. Afterward binding of GABA to GABA receptor in postsynaptic neuron action potentially inhibits dopamine cell firing in NAc. During chronic MMT, upregulation of opioid receptors intensifies the GABA release inhibition, over-activates PKA in MOR downstream signaling, and improves adaptation. On the other hand, we observed reduced *DNM1L* expression which, causes decline and blunting of receptor desensitization process and may develop tolerance. In response to reducing DA release in NAc dopaminergic synapse, we hypothesize that increased receptor expression of D1 and D2 consider as a compensation mechanism. Also, *COMT* reduction is another mechanism to preserve DA level in the presynaptic neuron in NA.

The upregulation of *DRD2* in MMT patients may represent a therapeutic prospect. *DRD2* through *GNAI1* could impact *RA1GAP*, which activates RAP1A and RAP1B by exchanging GDP with GTP. The activation of RAP1A follows this, and RAP1B functions on downstream paths, including PI3K-AKT signaling pathways and MAPK signaling pathways. Previously an in vivo study on cocaine administrated mice reported that a novel DA-PKA-Rap1-MAPK intracellular signaling mechanism is activated by *DRD1* could have regulated reward-related behavior. Rap1 stimulates MAPK (ERK) signaling and mediate neuronal excitability. [107] Another in vivo study showed that over-activation of *DRD2* impacts the A2aR-PKA-Rap1-MEK pathway and mediates aversive behavior. [108] On the other hand, D1R activation by methadone could shift from PKA to ERK intracellular signaling cascades.

ERK functioned as a crucial downstream kinase in the second messenger signaling pathway to mediate drug addiction, such as morphine. Elevation of ERK phosphorylation by addictive drugs has been reported previously. [109,110] which triggers the rewarding behavior. [111] Therefore, activation of ERK seems to be critical for drug cravings [112], and high persistent activity of ERK observed after a long period of withdrawal. Furthermore, in vivo studies demonstrated the involvement of ERK signaling in the reward drug-induced in the midbrain (MB). [113]

Increased ERK phosphorylation has been reported in drug reinforcement. [114] Moreover, the functional study reported the memory disruption by utilizing ERK inhibitors, which decreased the *ERK1/2* phosphorylation level. [115] These findings highlight the pivotal role of ERK in memory preserving.

While the ERK dephosphorylation exact mechanism is not adequately understood, several studies have focused on the ERK activation mechanism. [116] Targeting upstream signaling pathways mediated by ERK dephosphorylation could be a therapeutic approach that needs further investigation. Consequently, the dual-specificity phosphatase (DUSP) superfamily (ERK dephosphorylation enzymes) indicated benefits in diminishing addiction memory. [116]The amygdala microinfusion of *ERK1/2* inhibitor was previously utilized to decrease anxiety-related behavior in naive rats. [117] Also, the Y3214996, a potent, selective, ATP-competitive ERK inhibitor, was used in xenograft models (harboring ERK pathway alteration) to overcome resistance. This ERK inhibitor improved cancer treatment efficacy and demonstrated antitumor activity, recommended for future phase I clinical trials. [118] Also, another ERK inhibitor, ulixertinib on the clinical trial, was reported to be more efficient

and well-tolerated in different tumor types and introduced for use in combination therapy for MAPK-driven cancers. [119] However, there is a lack of any investigation for ERK inhibitor in OUD treatment strategy.

The list of most critical and related hubs in OUD pathogenesis pathways, which could be introduced as new potential therapeutic targets, is provided in the present study. Our study supports the therapeutic impacts of methadone on OUD through mimicking opioid signaling and upregulation of DRD2. Previously some evidence reports the involvement of ERK1/2 in the pathogenesis of synaptic plasticity and memory in addiction. [120,121] Also, a functional study on rats reported the elevation of ERK activity in NAc shell during morphine administration, and also ERK inhibitors prevent conditioned place preference. [122] ERK inhibitors or biomarkers (hubs) in the MAPK signaling pathway could be potential targets for further investigation.

The present study has some limitations. This study focuses on mRNA expression analysis; however, some mechanisms such as mRNA degradation and mRNA stability that could impact protein level are not excluded. Although, the cautious and considerate use of PBMCs as a surrogate for brain gene expression may additionally be warranted. Alternatively, further functional study with large sample size or comparative studies between tissue and blood on mammalian organisms would be informative. While future studies using a larger sample size are needed to validate our results. This case-control observational study provides essential information regarding genetic and receptor expression differences that may underlie the behavioral differences in OUD.

We propose that the D2 receptor has therapeutic potential for OUD, and using D2 agonist directly could replace methadone therapy to bypass opioidergic signaling. Also, ERK activation is a downstream pathway influenced during D2 upregulation. The inhibition of this pathway may also have a positive therapeutic impact in OUD. Further, studies investigating these two pathways can provide an efficient strategy for combating addiction while avoiding the side effects caused by the current therapeutic options.

5. Conclusions

In summary, methadone mimics the drug's behavior by significant upregulation of Opioid receptors. Downstream pathway induction due to DRD1 upregulation affects behavior patterns. COMT downregulation as a compensatory mechanism in response to reduced DA released during chronic opiate abuse in OUD could conceptualize dependency development in long-term treatment with methadone. Likewise, DRD5 downregulation is considered a barrier to depletion of reward-related incentive learning pathways in critical brain areas, which may develop dependency during long-term MMT. The ability of methadone to upregulate DRD2 is in line with potential therapeutic properties. A decrease in DRD3 during MMT was observed; therefore, DRD3 antagonists may be potentiated as an attractive target for strategies in the treatment of OUD. The resistance genes (RAB22A and DNM1L) are controversial in that they indicate the off-target deviation impact of long-term utilization of methadone and underlie tolerance. One of the advantages of our study is that we assessed the comprehensive gene profile simultaneously. Considering the text mining and data analysis of DA receptor gene expression in MMT patients, one hypothesis is that tolerance could be related to a synergistic correlation between DA receptors' activity and their downstream pathways. DRD2 modifiers (e.g., agonists) may function as a probable therapeutic target. Also, the present study reveals the significant role of the MAPK signaling pathway (specifically ERK activation) as an intersectional hub, underlying promising new directions for further research in OUD pathophysiology.

Abbreviations

Abbreviation	Expansion
MMT	Methadone Maintenance Therapy
DRD	Dopamine Receptor D
COMT	Catechol-O-Methyl Transferase
DNM1L	Dynamin 1 like
RAB22A	RAS-associated protein
PPI	Protein-protein interaction
MOR	Mu-Opioid Receptor
DOR	Delta-Opioid Receptor
KOR	Kappa-Opioid Receptor
MAPK	Mitogen-Activated Protein Kinase
UNODC	United Nations Office on Drugs and Crime
HIV	Human Immunodeficiency Virus
VTA	Ventral Tegmental Area
GABA	Gamma-Aminobutyric Acid
DA	Dopamine
CNS	Central Nervous System
PBL	Peripheral Blood Lymphocyte
OUD	Opioid Use Disorder
PBMCs	Peripheral Blood Mononuclear Cells
EDTA	Ethylenediamine-Tetraacetic Acid
PBS	Phosphate-Buffered Saline
mRNA	messenger Ribonucleic acid
cDNA	Complimentary Deoxyribonucleic acid
REST	Relative Expression Software Tool
GEO	Gene Expression mnibus
MCODE	Molecular Complex Detection
DAVID	Database for Annotation, Visualization and Integrated Discovery
GO	Gene Ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
Nac	Nucleus Accumbens
BLA	Basolateral Amygdala
MA	Methamphetamine
CTX	Cerebral Cortex
LTP	Long-Term Potentiation

MBH	Medial Basal Hypothalamus
SUD	Substance Use Disorder
cAMP	Cyclic Adenosine Monophosphate
PKA	Protein Kinase A
CREB	cAMP Response Element-Binding
LC	Locus Coeruleus
MB	Midbrain
DUSP	Dual-Specificity Phosphatase

Declarations

Consent for Publication

All participants read and signed the consent form.

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Conflict of Interest statement:

The authors declared no conflict of interest.

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Data availability

The data that supports the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request.

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Figures

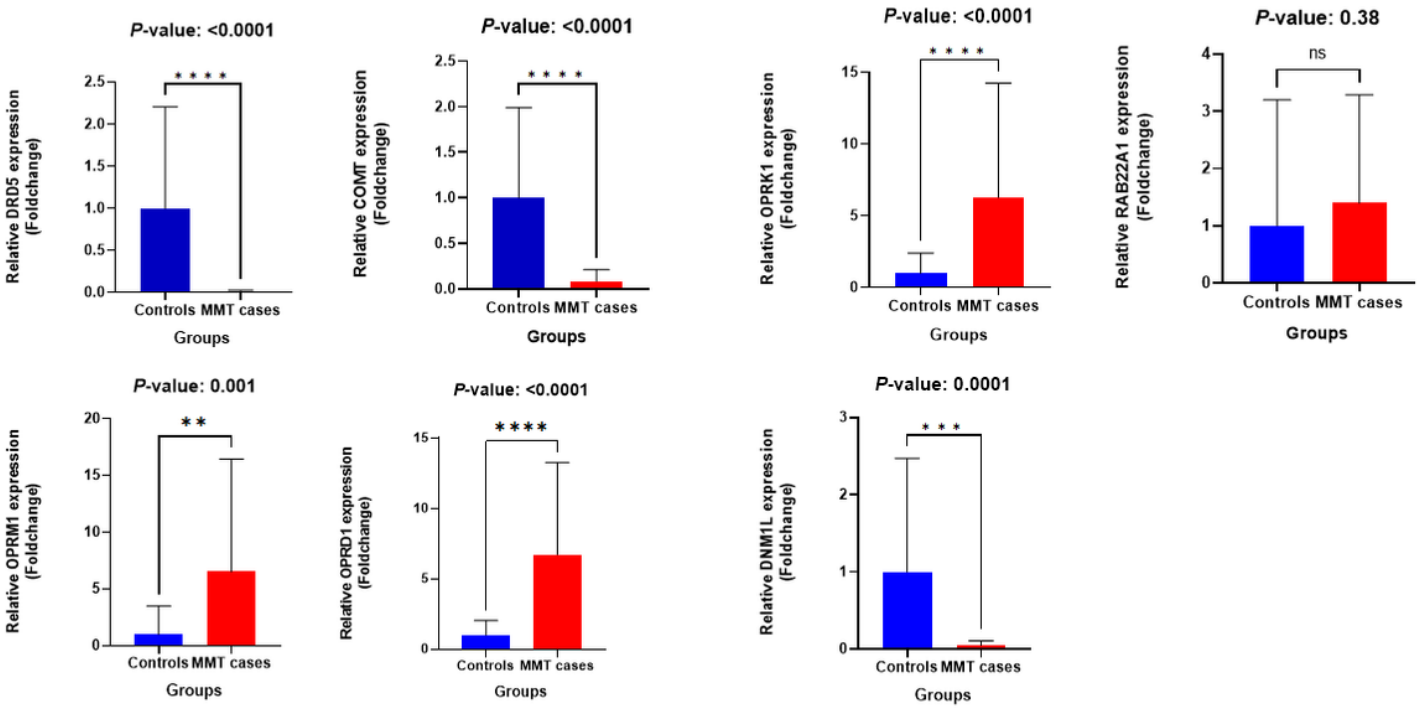

Figure 1

Shows the dopamine D1, D2, D3, D4 & D5 receptors and *COMT* and *OPRM1* & *OPRD1* and *OPRK1* and also *RAB22A* & *DNM1L* mRNA expression in the peripheral blood lymphocytes of the control and methadone maintenance treated (MMT) individuals. Bars represent fold differences of mean normalized expression values ± S.E.M (DRD1; n=38, D2 to D5 & *COMT* & *OPRM1* & *OPRD1* & *OPRK1* & *RAB22A* and *DNM1L*;n=40).

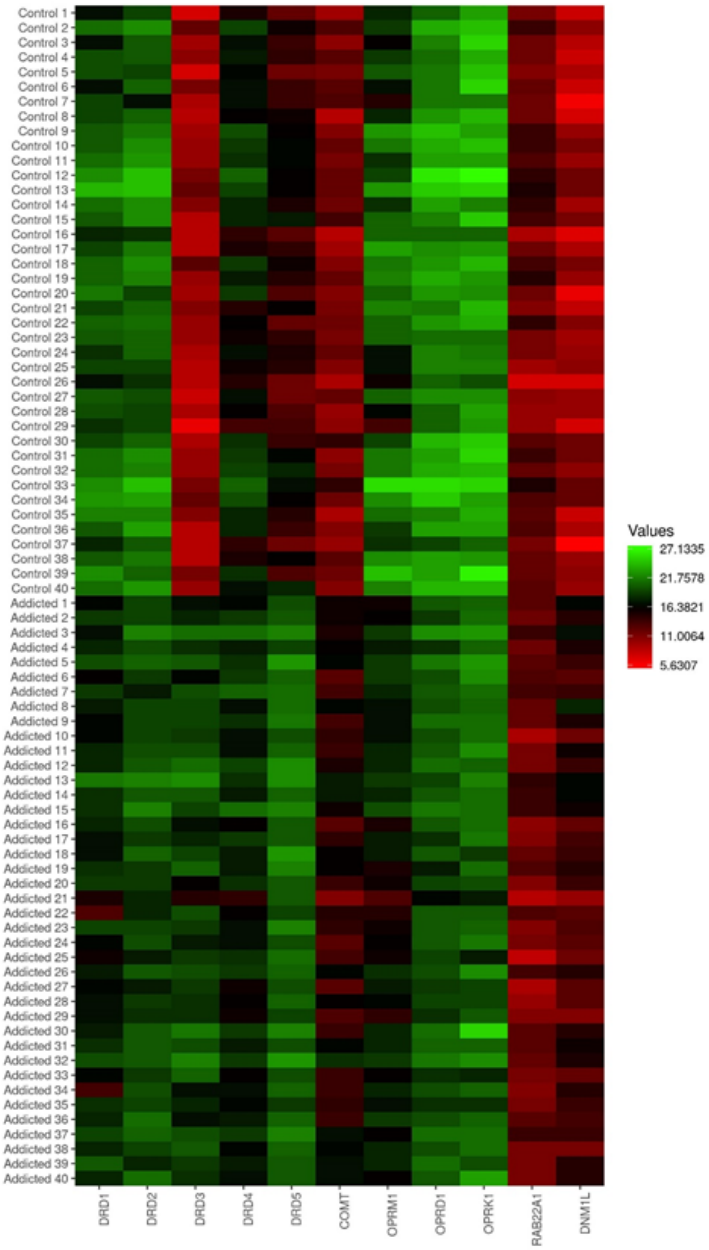

Figure 2

The heatmap presents the relative DeltaCt values of *DRD1* to *DRD5* and *COMT* enzyme & *OPRM1* & *OPRD1* & *OPRK1* & *RAB22A* and *DNM1L* between two groups (Control vs Methadone maintenance). The lower the DeltaCt the higher the gene expression. Visual distinction between the two groups is evident. See legend for details.

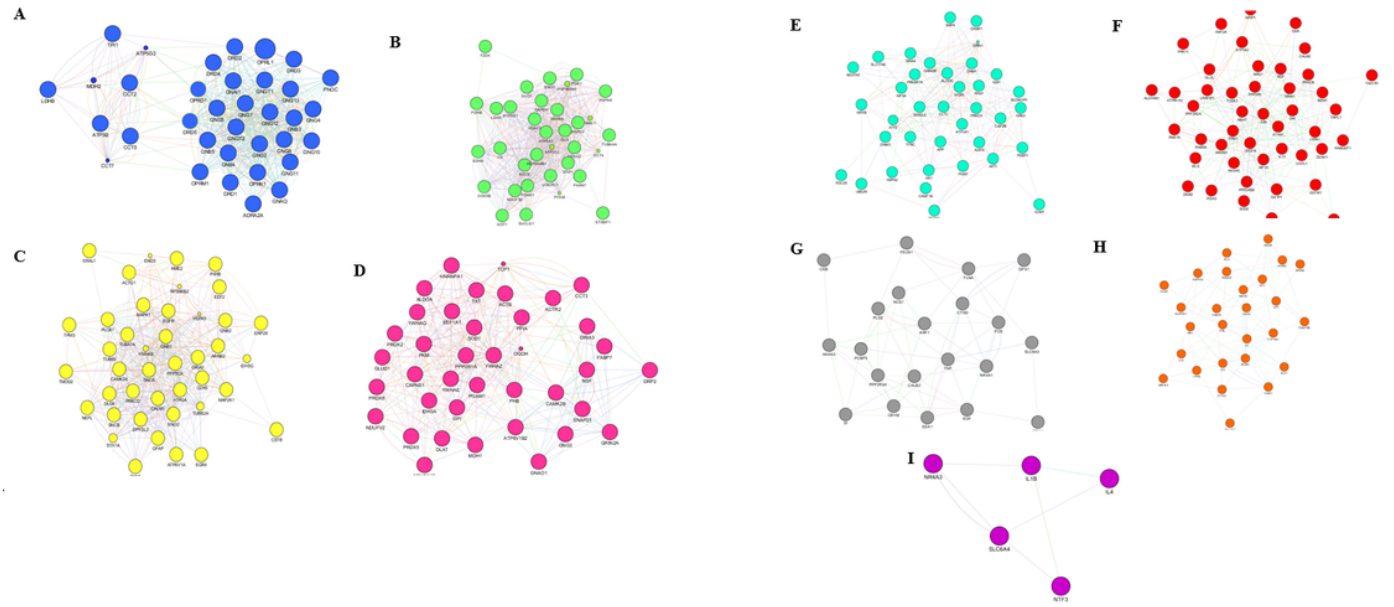

Figure 3

Clusters of intersection network identified by MCODE plugin. A Cluster 1, B Cluster 2, C Cluster 3, D Cluster 4, E Cluster 5, F Cluster 6, G Cluster 7, H Cluster 8, I Cluster 9.

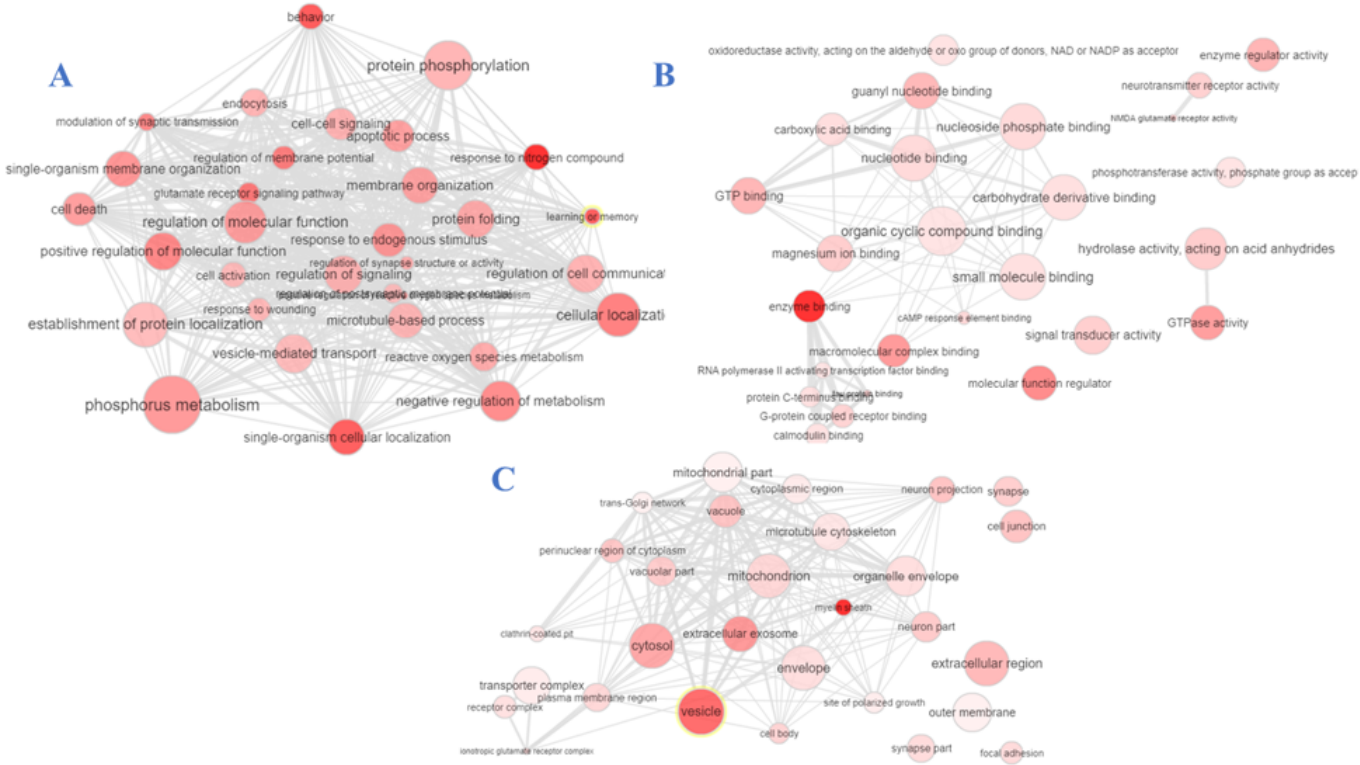

Figure 4

GO terms networks of biological processes (A), molecular functions (B), cellular components (C) and their p-values associated with ranked gene set for Addiction. Each node represents a biological process of each gene. Node color indicates the p-values of each GO term in this intersection (darker = more abundant). Node size indicates the generality of each GO term (smaller = more specific). Edges represent the 3% of the strongest GO term pairwise similarities. The y-Files Organic Layout algorithm was applied to display the topology of the network.

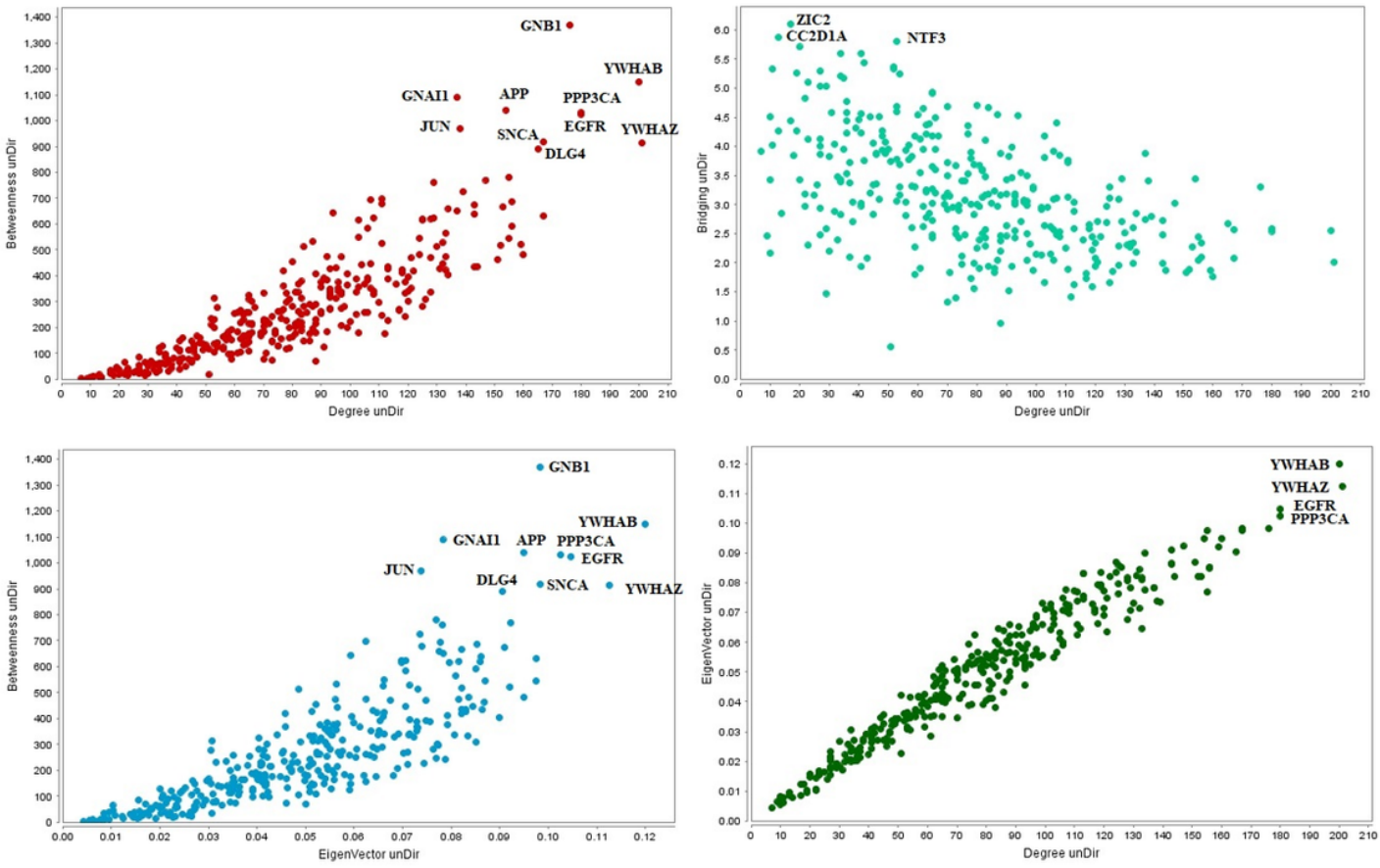

Figure 5

Scatter plots of the centralities parameters.

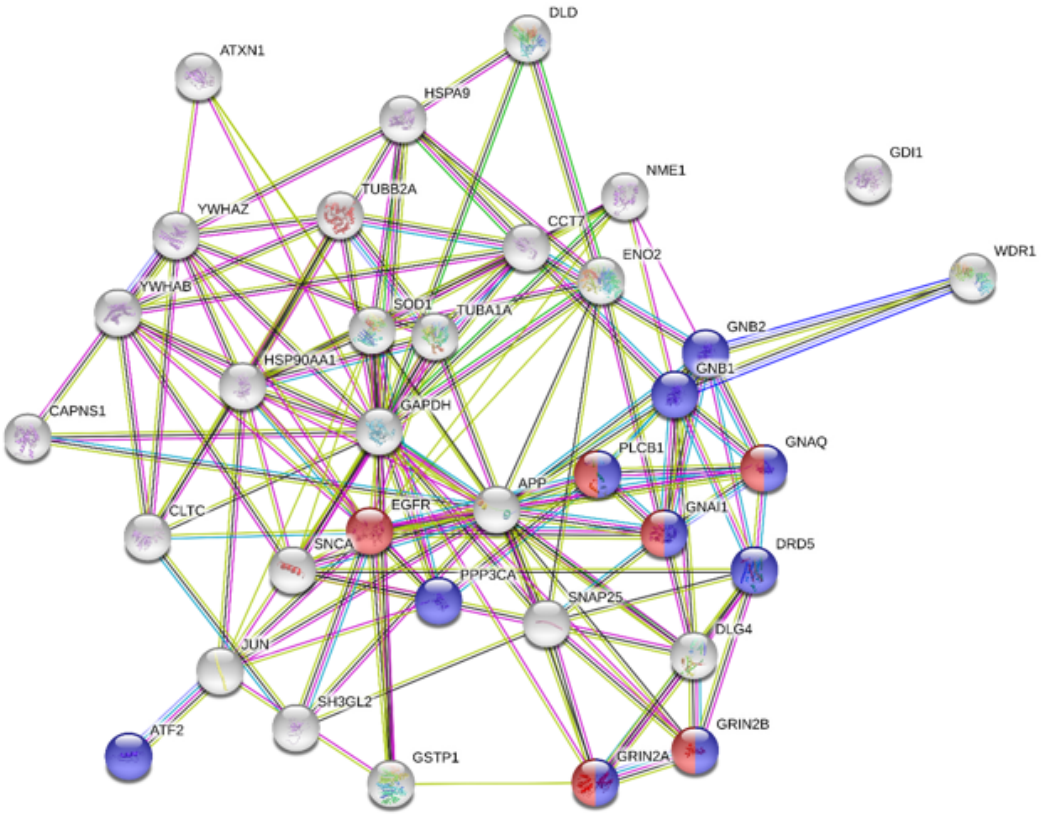

Figure 6

the nodes of the 2nd extended PPI network involved in the KEGG pathways of Dopaminergic synapse and Rap1 signaling were colored in blue and red, respectively.

Supplementary Files

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- GraphicalAbstract.docx
- S1.Enrichmentanalysisinput..xlsx
- S2.GOterms..xlsx
- S3.Expandedgenesetsinput..xlsx
- S4.Centiscapeconsistsofnetworktopologyparameter..xlsx
- S5.Networktopologicalparametersranking..xlsx